

Evidence for Antibacterial Activity in Haemolymph of Diplopoda: Preliminary Results

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ABSTRACT

In a screening bioassay, the antibacterial activity in haemolymph from eight species of millipedes (*Megaphyllum projectum kochi*, *Ommatolulus sabulosus*, *Unciger foetidus*, *Polydesmus complanatus*, *Glomeris connexa*, *Strongylosoma pallipes* auct., *Leptoiulus proximus*, *Oxidus gracilis*) was compared with cell-free antibacterial immunity of *Galleria mellonella* pupae. The only millipedes where lysozyme was not constantly detectable are *M. projectum kochi* and *S. pallipes*. In others, the low constitutive titre of lysozyme was unaffected by injections with *Enterobacter cloacae* B12 or nutrient broth. In *Galleria*, as for the majority of insects, such previously present antibacterial activity increased markedly after preinjection of the pupae with *E. cloacae* or sterile broth. In pupae of *Galleria*, the antibacterial activity of cecropin-like type is induced by *E. cloacae* as well as by non-living material, the broth. A trace activity against *Escherichia coli* D31 was present in untreated *Unciger*, but this titre did not increase after bacterial inoculation. Haemolymph from *M. projectum kochi*, *P. complanatus* and *O. gracilis* investigated 2 days after injections of either broth or *E. cloacae* did not show any inducible antibacterial activity. Injections of *E. cloacae* into *Ommatolulus*, but broth into *Glomeris*, induce a measurable antibacterial activity against *E. coli* D31.

RÉSUMÉ

Mise en évidence d'une activité antibactérienne dans l'hémolymphe de Diplopoïdes.

Chez huit espèces de diplopoïdes (*Megaphyllum projectum kochi*, *Ommatolulus sabulosus*, *Unciger foetidus*, *Polydesmus complanatus*, *Glomeris connexa*, *Strongylosoma pallipes* auct., *Leptoiulus proximus*, *Oxidus gracilis*), l'activité antibactérienne de l'hémolymphe a été comparée à l'immunité antibactérienne des pupes de *Galleria mellonella*. Les seuls diplopoïdes chez lesquels les lysozymes ne sont pas constamment décelables sont *M. projectum kochi* et *S. pallipes*. Chez les autres espèces, on ne note aucun effet à la suite d'injections de *Enterobacter cloacae* B12 ou de milieu de culture nutritif. Chez *Galleria*, comme chez la plupart des insectes, l'activité antibactérienne s'accroît significativement après injection de la pupe par *E. cloacae* ou un milieu stérile. Chez la pupe de *Galleria*, l'activité antibactérienne de type cécropine est induite aussi bien par *E. cloacae* que par un milieu non-vivant. Une trace d'activité anti-*Escherichia coli* D31 est présente chez *Unciger* non traité, mais ce taux ne s'accroît pas après une inoculation bactérienne. L'hémolymphe de *M. projectum kochi*, *P. complanatus* et *O. gracilis*, étudiés deux jours après injection soit de milieu de culture, soit de *E. cloacae*, ne montre aucune activité antibactérienne décelable. Des injections de *E. cloacae* chez *Ommatolulus* induisent une activité antibactérienne mesurable à l'encontre de *E. coli* D31 alors que cet effet est produit par le milieu de culture chez *Glomeris*.

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INTRODUCTION

Invertebrates can defend themselves against bacterial infections by both cellular (SALT, 1970) and humoral (CHADWICK, 1975) defence mechanisms. A potent humoral immune system that can be induced by an infection with live non-pathogenic bacteria or injections of abiotic foreign bodies is specially well characterized in pupae of Lepidoptera (BOMAN & HULTMARK, 1987) which respond to the infection with *Enterobacter cloacae* by the synthesis of several classes of immune proteins. Humoral immunity in lepidopterans and other holometabolous insects is due mainly to the antibacterial action of lysozyme (MOHRING & MESSNER, 1968) and a new class of small basic polypeptides, the cecropins (BOMAN & HULTMARK, 1987).

The presence of lysozyme in normal and immunized arthropods has been reported in phylogenetically distant species of invertebrates (GOTZ & TRENCZEK, 1991), including myriapods (XYLANDER & NEVERMANN, 1990). Of inducible bactericidal immune proteins that are de novo synthesized by several orders of Insecta, cecropins produced in *Hyalophora cecropia* and other lepidopterans are the first antibacterial factors well defined biochemically.

In this paper we compared the antibacterial activity in haemolymph from eight species of millipedes with the pupal *Galleria mellonella* immune system, using techniques for antibacterial activity assays developed in the study of cell-free insect immune responses.

MATERIALS AND METHODS

Millipedes

Using a cup plate agar-diffusion assay technique, antibacterial activities of lysozyme and cecropins were detected in native (non-immune) and immune haemolymph of diplopod species: *Megaphyllum projectum kochi* (Verhoeff), *Ommatoiulus sabulosus* (L.), *Unciger foetidus* (C. L. Koch), *Polydesmus complanatus* (L.), *Glomeris connexa* C. L. Koch, *Strongylosoma pallipes* (auct.), *Leptoiulus proximus* (Nemec), *Oxidus gracilis* (C. L. Koch). All the millipedes investigated live within woodland litter and soil as their natural habitat. In laboratory, the specimens caged in glass vessels fed plant litter in varying stages of decomposition. Animals were maintained at 12° C until bleeding for bioassays of antibacterial activities.

Induction of immune response and sampling of haemolymphs

For immunization, the millipedes were injected into the abdominal haemocoel with either live, log phase *Enterobacter cloacae* (0.6 x 10⁴ bacteria per specimen) or sterile nutrient broth (3.0 ml), an abiotic soluble foreign molecule. By the same way, two day old pupae of *Galleria mellonella* (Lepidoptera, Pyralidae) taken out of their cocoons were inoculated with immunizing bacteria or nutrient broth. Fully vigorous, unwounded pupae were served as a control because of available already evidence on antibacterial immune proteins active in insect immunity (JAROSZ, 1993). Millipedes treated with foreign bodies were incubated for 48 hours at 12°C, but pupae of *Galleria* at 26°C. Haemolymph from millipedes were obtained after incision of the intersegmental abdominal cuticle, using a sterile glass micropipette. Only small volumes of blood may be collected from each individual, but trace amounts of haemolymph obtained from most of the diplopod species herein investigated were quite sufficient to assay the antibacterial activities in a thin agar layer with the wells of 0.7 mm in diameter.

Bioassays for antibacterial activities

Lysozyme activity (E. C. 3.2.1.17; endo- β -1-4/-N-acetylmuramide glycanohydrolase) was determined in an inhibition zone assay around the well, using freeze-dried *Micrococcus luteus* incorporated into an agar medium at a concentration of 1.0 mg/ml, according to MOHRIG & MESSNER (1968). The test for haemolymph lysozyme activity was conducted in 0.066 M Sorensen buffer (pH 6.4) with 1.0% agarose and 70 mg/ml streptomycin sulfate to inhibit bacterial contaminations.

Bactericidal activity of cecropins provoked in pupal haemolymph of *Galleria* was quantified as a diameter of the lysis zone around the well in a thin agar layer inoculated with an overnight culture of *Escherichia coli* (about 0.3 x 10⁵ log phase cells per ml), strain D31 sensitive to cecropin-like activity. Haemolymph samples loaded into 0.7 mm diameter wells cut in the soft (0.7%) agar medium, were incubated at 28°C for 36 hours. Agar medium for assay of cecropin activity (but not for lysozyme) contained a trace of phenylthiourea to prevent melanization due to phenoloxidase activity.

RESULTS

The antibacterial activity in haemolymph of non-immunized and *E. cloacae*-, or broth-injected millipedes was compared with the activity of lysozyme and that of cecropins induced in *Galleria* pupae. Though considerable differences in haemolymph lysozyme activity of different individuals and different diplopod species were noticed, the normally low lysozyme activity was unaffected by *E. cloacae* β 12 or broth. The normally low lysozyme titer present in untreated *O. sabulosus*, *U. foetidus* and *P. complanatus* did not increased in specimens injected with foreign bodies (Table 1). No differences in haemolymph activity were noted between immunized and non-immunized diplopods. In our screening experiments, haemolymph lysozyme was not present in untreated *M. projectum kochi* and *S. pallipes* but a relatively high innate activity of lysozyme was found in *G. connexa* and *L. proximus*. Injections of foreign materials into body cavity of the millipede *O. gracilis* did not increase the hardly any detectable the constitutive antibacterial activity of haemolymph lysozyme. In contrast, the normally high innate lysozyme titer in *Galleria* pupae becomes elevated after bacterial infections (0.4×10^5 *E. cloacae*) so much as after injections of broth.

TABLE 1. — Antibacterial activity of lysozyme in haemolymph of non-immunized millipedes and those immunized with *Enterobacter cloacae* or sterile nutrient broth. Tr; trace activity; lysis zone diameter less than 1.0 mm (diameter of wells; 0.7 mm). “-”; not examined because of difficulty in collecting the haemolymph sample.

| Diplopod species | Haemolymph lysozyme activity; lysis zone diameter (mm) | | |
|-------------------------------------|--|-----------------------------|------------------------|
| | Non-immunized | Specimens immunized with: | |
| | | <i>Enterobacter cloacae</i> | Sterile nutrient broth |
| <i>Megaphyllum projectum kochi</i> | 0 | 0 | 0 |
| <i>Ommatoiulus sabulosus</i> | 1.5 | 1.5 | 1.5 |
| <i>Unciger foetidus</i> | 1.5 | 1.8 | 1.6 |
| <i>Polydesmus complanatus</i> | 1.7 | 1.7 | 1.8 |
| <i>Glomeris connexa</i> | 5.0 | 5.5 | 5.0 |
| <i>Strongylosoma pallipes</i> auct. | 0 | - | - |
| <i>Leptoiulus proximus</i> | 4.0 | - | - |
| <i>Oxidus gracilis</i> | Tr | Tr | Tr |
| <i>Galleria mellonella</i> | 8.0 | 10.5 | 10.0 |

In pupae of *Galleria*, cecropin antibacterial activity could normally be provoked by both living *E. cloacae* and sterile broth. Injections of broth (3.0 ml per animal) into body cavity of *G. connexa* can induce within 2 days a measurable antibacterial activity against *E. coli* D31 (Table 2). A similar but less pronounced antibacterial activity could be generated in *O. sabulosus* treated with *E. cloacae*. Other millipedes injected with *E. cloacae* (or broth) did not develop antibacterial activity against *E. coli*. Trace activity directed against *E. coli* was found in immunized and non-immunized *U. foetidus*. Further investigations are, however, needed to elucidate if the antibacterial activity appeared in immunized *G. connexa* and *O. sabulosus* could tentatively be classified to an inducible activity of cecropin-like type. Antibacterial activity against *E. coli* in the haemolymph of *G. connexa* and *O. sabulosus* might be detected only in some

individuals. These inconsistent results may partly be caused by the difficulty in collecting enough haemolymph from these millipedes. Furthermore, many specimens of millipedes treated with *E. cloacae* died just one day after bacterial injections.

TABLE 2. — Efforts to induce the cecropin-like antibacterial activity in haemolymph of Diplopoda by bacterial infections with *Enterobacter cloacae* or inoculations of the millipedes with sterile nutrient broth. Tr; trace activity, lysis zone diameter of *E. coli* D31 less than 1.0 mm (diameter of the well, 0.7 mm). “-”; not examined.

| Diplopod species | Cecropin-like activity; lysis zone diameter of <i>E. coli</i> (mm) | | |
|-------------------------------------|--|-----------------------------|------------------------|
| | Non-immunized | Specimens immunized with: | |
| | | <i>Enterobacter cloacae</i> | Sterile nutrient broth |
| <i>Megaphyllum projectum kochi</i> | 0 | 0 | 0 |
| <i>Ommatoiulus sabulosus</i> | 0 | 1.7 | Tr |
| <i>Unciger foetidus</i> | Tr | Tr | Tr |
| <i>Polydesmus complanatus</i> | 0 | 0 | 0 |
| <i>Glomeris connexa</i> | 0 | Tr | 4.5 |
| <i>Strongylosoma pallipes</i> auct. | 0 | - | - |
| <i>Leptoiulus proximus</i> | 0 | - | - |
| <i>Oxidus gracilis</i> | 0 | 0 | 0 |
| <i>Galleria mellonella</i> | 0 | 8.5 | 8.0 |

DISCUSSION

Despite of the progress in insect immunology, only scant evidence are still available about antibacterial substances, both innate and inducible, conditioning humoral immunity in myriapods. Intensive research activity of the last few years has led to the identification of new and interesting groups of peptides with antibacterial activity. It seems that their occurrence is not restricted to insects. XYLANDER & NEVERMANN (1990) have described at least two antibacterial substances in haemolymph of Diplopoda; one substance is lysozyme but the other one is different from lysozyme. Activity against living *Micrococcus luteus* increased after inoculation with *E. cloacae* B12 in two diplopod species, *Rhapidostreptus virgator* and *Chicobolus* sp. Independently of the bacterial strain used as an immunizing agent, growth of *E. cloacae*, but not *E. coli*, was inhibited by haemolymph (XYLANDER & NEVERMANN, 1990). Thus far, the activity against *E. coli* was detected only in haemolymph from the millipede *Triaenostrepus triodus* (VAN DER WALT *et al.*, 1990), and in haemolymph of immunized diplopods *Ommatoiulus sabulosus* and *Glomeris connexa* (Table 2).

CONCLUSION

The low innate haemolymph lysozyme titer that increases drastically in insects invaded with non-pathogenic bacteria (MOHRIG & MESSNER, 1968) and inducible antibacterial immune proteins like cecropins that are synthesized in the fat body of several insect species (BOMAN & HULTMARK, 1987), but rather absent in millipedes, could confirm the suggestions of RAVINDRANATH (1973) and NEVERMANN & XYLANDER (1992), concerning the crucial role of cellular immune responses in antibacterial defences of Diplopoda. The phenoloxidase system, that is considered to be one of the main systems of immune defense in arthropods being responsible for foreign recognition, killing of microbial invaders, encapsulation of parasites and

wound healing helps the haemocytic reactions in diplopods since the melanization is found at wound margins, in the haemocytic wound closure and in haemocytic capsules around foreign bodies (XYLANDER & BOGUSCH, 1992).

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